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Engineering an Inward Proton Transport from a Bacterial Sensor Rhodopsin

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Abstract: ATP is synthesized by an enzyme that utilizes proton motive force, and thus, nature has created various proton pumps. The best-understood proton pump is bacteriorhodopsin (BR), an outward-directed, light-driven proton pump in *Halobacterium salinarum*. Many archaeal and eubacterial rhodopsins are now known to show similar proton transport activity. We previously converted BR into an inward-directed chloride ion pump, but an inward proton pump has never been created. Proton pumps must have a specific mechanism to exclude transport in the reverse direction in order to maintain a proton gradient, and in the case of BR, a highly hydrophobic cytoplasmic domain may constitute such machinery. Here we report that an inward-directed proton transport can be engineered from a bacterial rhodopsin by a single amino acid replacement. *Anabaena* sensory rhodopsin (ASR) is a photochromic sensor in freshwater cyanobacteria that possesses little proton pump activity. When we replaced Asp217 in the cytoplasmic domain (a distance of ∼15 Å from the retinal chromophore) by Glu, ASR exhibited an inward proton transport activity driven by absorption of a single photon. FTIR spectra clearly showed an increased proton affinity for Glu217, which presumably controls the unusual directionality opposite to that in normal proton pumps.

1. Introduction

In bacteriorhodopsin (BR), a retinal chromophore is located at the center of the membrane, and the hydrophobicity is different between the cytoplasmic and extracellular domains.[1] The cytoplasmic domain is highly hydrophobic, whereas the extracellular domain is composed of charged and polar amino acids that form a hydrogen-bonding network. Figure 1A shows the presence of seven to eight water molecules in the extracellular domain but only two water molecules in the cytoplasmic domain. Such an asymmetric hydrogen-bonded network could be the reason for unidirectional proton transport in BR, where proton transfer to the extracellular side occurs in 10^{-5} s and is followed by reprotonation through a transiently formed proton pathway in the cytoplasmic domain on a longer time scale (10^{-4} to 10^{-3} s).[2] We previously converted BR into an inward-directed chloride ion pump,[3] but an inward proton pump has never been created. It may be difficult to design an inward proton pump from "normal" outward proton pumps.

Anabaena sensory rhodopsin (ASR) is an archaeal-type rhodopsin found in *Anabaena* (*Nostoc*) sp. PCC7120, a freshwater cyanobacterium. ASR does not show proton pump activity, and as it forms a single operon with a soluble protein of 14 kDa,[4] it has been suggested that ASR is a photochromic sensor activating the 14 kDa transducer protein at the cytoplasmic surface.[5,6] The X-ray crystallographic structure of ASR[6] has an α-helical arrangement similar to that of BR but a very different hydrogen-bonded network. Figure 1B shows that in ASR, both the extracellular and cytoplasmic domains contain five water molecules and form hydrogen-bonded networks. Consistent with the hydrogen-bonded network in the cytoplasmic domain, an unusual proton transfer has been found in this protein. Shi et al.[7] reported reverse proton transfer from the Schiff base to Asp217 in the cytoplasmic domain, whereas Sineshchekov et al.[8] reported that the direction of this proton transfer is dependent on C-terminus truncation. Therefore, proton conduction in ASR remains unclear at present, although proton conductivity toward the cytoplasmic domain appears possible. This suggests the potential to design an inward proton pump involving ASR, and we have indeed achieved this. A single amino acid replacement of Asp217 to Glu confers inward proton transport activity to ASR.

2. Materials and Methods

Sample Preparation. In the present study, we prepared C-terminally truncated and full-length ASR according to a method

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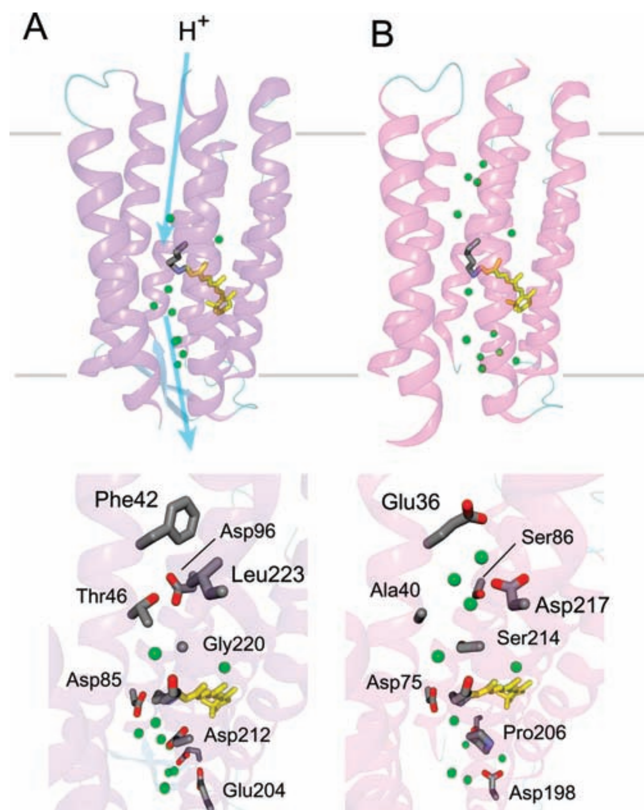

Figure 1. X-ray crystallographic structures of (A) BR[1] and (B) ASR.[6] The top and bottom panels represent views from the membrane plane and the cytoplasmic side, respectively. In the top panel, the top and bottom regions correspond to the cytoplasmic and extracellular sides, respectively. The retinal chromophore is colored yellow, and green spheres represent internal water molecules. BR (A) is a light-driven, outward-directed proton pump, where Asp85 accepts a proton from the Schiff base and Asp96 donates a proton to the Schiff base.[1] No proton pump activity has been reported for ASR.[4] ASR (B) is a sensor protein that activates a soluble protein at the cytoplasmic surface, and it has been reported that the Schiff base proton is transferred to Asp217 in the cytoplasmic region.[7,8] Polar amino acids as well as five water molecules in the cytoplasmic region are characteristic of ASR and must be advantageous for primary proton transfer that may be important for activation of the soluble transducer protein. This provides an experimental basis for designing an inward proton transport in the present study.

described previously.[4,5,9] The D217E mutant was designed on the basis of wild-type (WT) ASR, which was produced by a two-step megaprimer PCR method[10] using the oligonucleotide 5′-ACG TAA GCC GTG TAA TTC CAG AAA ACT AAA TCC-3′ (Hokkaido System Science, Japan). The final PCR products were cloned into plasmid pKJ606,[11] derived from pMS107, by replacing the original insert with XbaI/NotI digestion. After ligation, the plasmids were transformed in *Escherichia coli* strain JM109. All of the mutations were confirmed by DNA sequencing (Hokkaido System Science).

E. coli strain BL21 (Stratagene) was transformed by introducing pMS107-derivative plasmid, which encodes WT, D217E, and D217N ASR and WT *Gloeobacter* rhodopsin (GR) and was grown in 2× YT medium in the presence of ampicillin (50 mg/mL) at 37 °C. Three hours after isopropyl-β-D-thiogalactopyranoside (IPTG) induction with the addition of 10 mM all-trans retinal, pink-colored cells were harvested by centrifugation at 3600*g* for 15 min at 4 °C and suspended in 10 mL of 30 mM Tris-HCl (pH 8.0) and 20% sucrose. Next, a sphaeroplast sample was prepared as follows. Lysozyme (100 mg) was added and stirred gently at room temperature for 15 min. Sphaeroplasts were spun down at 3600*g* for 15 min at room temperature, resuspended in 100 mM potassium phosphate (KPi) (pH 7.0), 20 mM $MgSO_4 \cdot 7H_2O$, 20% sucrose, and 4 mg of DNase (400 μL), and injected slowly using a 1 mL syringe (18 gauge needle) into 200 mL of a rapidly stirring (200 rpm) solution of 50 mM KPi (pH 7.0) at 37 °C. After the mixture was gently stirred for 15 min, sodium EDTA was added to a final concentration of 10 mM, and the solution was stirred for another 15 min. The right side-out of sphaeroplast vesicles was collected at 3600*g* for 15 min at 4 °C (CF16RX centrifuge, Hitachi, Japan), washed with 10 mL of unbuffered solution (150 mM NaCl, 50 mM $MgSO_4 \cdot 7H_2O$), and spun down at 3600*g* for 15 min at 4 °C. The sphaeroplast suspension (pH 6.3−6.7) was used for the measurements of proton pump activity.

For spectroscopic and HPLC analysis, the harvested samples after IPTG induction were sonicated, solubilized by 1% dodecyl maltoside (DM), and purified by a Ni^{2+}−NTA column as described previously.[12,13] The purified ASR samples in 0.1% DM solution [300 mM NaCl, 50 mM Tris-HCl, 150 mM imidazole (pH 7.0)] were used for UV−vis spectroscopy and HPLC analysis.[13] For FTIR spectroscopy, the purified ASR was then reconstituted into phosphatidylcholine (PC) liposomes by removing the detergent with Biobeads, where the molar ratio of the added PC to ASR was 30:1.[12,13] The liposomes were washed three times with a buffer [2 mM sodium borate (pH 9.0)].

Light-Induced pH Changes. The proton transport activity of each protein was measured by monitoring pH changes in a sphaeroplast suspension by a glass electrode.[14] Sphaeroplasts containing ASR or GR protein in unbuffered solution (50 mM $MgSO_4$, 150 mM NaCl) were illuminated at >500 nm through a glass filter (AGC Techno Glass Y-52, Japan), and the pH value changes were monitored (F-55 pH meter, Horiba, Japan). The light source was a 1 kW tungsten−halogen projector lamp (Master HILUX-HR, Rikagaku, Japan). The samples were then illuminated after addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to a final concentration of 10 μM. Proton transport activities were calculated by adding 10 μL of 0.01 N HCl to the suspension. The amount of protein in the sphaeroplast suspension was estimated by measuring absorption spectra after solubilizing the protein with 1% DM. Three to six independent measurements were averaged to obtain the initial rate of proton transport.

For the light intensity dependence of the initial rate of proton transport, 50, 25, 10, and 1% neutral density filters were used. The action spectrum for the proton transport activity was measured with monochromatic light (half width 10 nm) from interference filters (03FIV107, 006, 109, 008, 117, 018, and 119, Melles Griot, USA), where the wavelength-dependent light intensity of the tungsten−halogen lamp was calibrated using a CCD linear detector (PMA-12, Hamamatsu Photonics, Japan).

UV−Vis Spectroscopy. Absorption spectra of WT and D217E ASR were measured in 0.1% DM solution [300 mM NaCl, 50 mM Tris-HCl, 150 mM imidazole, (pH 7.0)] at 20 °C by use of a Shimadzu UV-2400PC UV−vis spectrometer. The samples were illuminated with >500 nm light for 4 s at 277 K, and the decay kinetics of the M intermediate was monitored at 388 nm.

HPLC Analysis. HPLC analysis was performed as described previously.[13] Dark-adapted ASR was prepared by keeping the samples in the dark overnight at 4 °C. Extraction of retinal oxime from the sample was carried out using hexane after denaturation with methanol and 500 mM hydroxylamine at 4 °C.

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FTIR Spectroscopy. FTIR spectroscopy was performed as described previously.[12,13] A 40 μL aliquot of the PC liposomes was deposited on a BaF_2 window with a diameter of 18 mm and dried in a glass vessel that was evacuated by an aspirator. The ASR film sample was hydrated with 1 μL of H_2O or D_2O before the measurements. Next, the sample was placed in a cryostat (DN-1704, Oxford, U.K.) mounted in the FTIR spectrometer (FTS-40, Bio-Rad, USA). The cryostat was equipped with a temperature controller (Oxford ITC-4), and the temperature was regulated with 0.1 K precision. All of the experimental procedures until setting of the samples were performed in the dark or under dim red light (>670 nm).

Illumination with >500 nm light at 230 K for 4 s converted ASR to ASR_M. Each difference spectrum was calculated from two spectra constructed from 64 interferograms taken before and after the illumination. Three difference spectra obtained in this way were averaged to produce the ASR_M-minus-ASR spectrum.

3. Results and Discussion

The WT and mutant proteins of ASR were expressed in *E. coli*. We used full-length ASR here, but C-terminally truncated ASR was also prepared for the WT. For proton transport measurements, we prepared sphaeroplast vesicles by removing the cell wall by lysozyme treatment. Illumination caused a net inward transport of protons for D217E ASR, resulting in an alkaline pH of the medium (Figure 2, top panel). The observation of no transport after addition of CCCP (Figure 2, top panel) and in the absence of retinal (Figure 2, second panel) clearly demonstrates that the D217E mutant functions as a light-driven inward proton transport.[15] Inward proton transport activity was also observed for the WT ASR (Figure 2, third panel), but the transport activity was much less than that for D217E ASR. Although the previous photocurrent measurements reported that the directions of the initial proton transfer are inward and outward for truncated and full-length WT ASR, respectively,[8] the small inward proton transport observed in this study was similar for the truncated and full-length WT samples (data not shown). The proton transport activity was negligible for D217N ASR (Figure 2, fourth panel). We also measured an outward-directed proton pump as a control. The bottom panel of Figure 2 shows the result with GR found in a primitive cyanobacterium. In sphaeroplast vesicles containing GR, light caused outward proton transport, as for BR.

It should be noted that light-driven inward proton transport associated with two-photon reactions has been reported for the D85N mutant of BR in films attached to planar lipid bilayers,[16] where the molecular mechanism has not been well-established. It appears that absorption of a second photon by the deprotonated Schiff base is necessary for the inward proton transport. To test such a possibility in the present case, we measured proton transport activity with different light intensities. Figure 3A clearly shows a linear relationship, indicating that the inward proton transport in D217E ASR is driven by a single-photon reaction. The action spectrum for proton transport activity, measured with monochromatic light, resembles the absorption spectrum of D217E ASR (Figure 3B). The initial slope for the inward proton transport in D217E ASR was 15.1 $\pm$ 4.0 H^+ $protein^{-1}$ min^{-1}, which is about half that in GR (Figure 2, bottom panel) and 15 times smaller than that in BR.[17] Figure 3C shows that the inward proton transport by D217E ASR is pH-independent between pH 6.5 and 8.0.

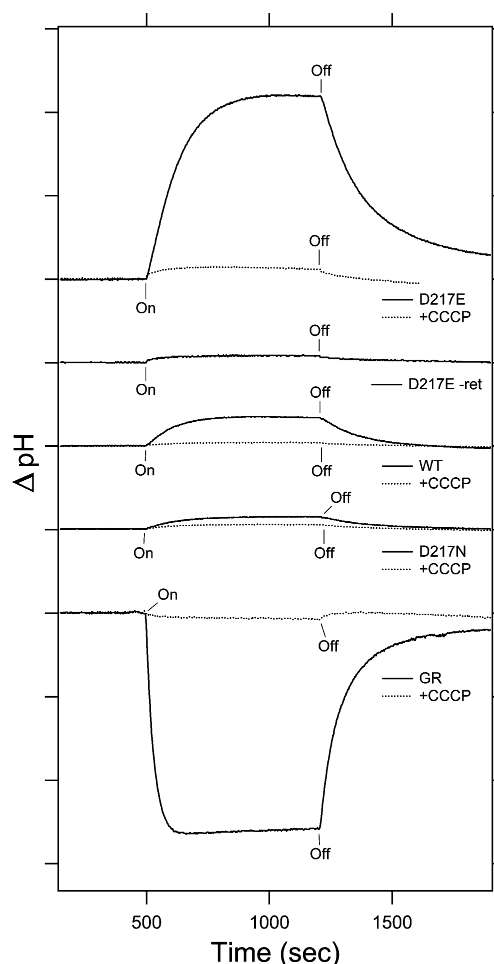

Figure 2. Light-driven proton transport activity in sphaeroplast vesicles containing ASR or GR (50 mM $MgSO_4$, 150 mM NaCl, initial pH ~6.5). "On" and "Off" indicate the onset and offset of illumination (with yellow light, >500 nm), respectively, and positive signal corresponds to a decrease in pH (inward proton transport). The signal amplitude of each protein was normalized to its absorption after the transport activity measurement, allowing that the transport activity to be numerically compared among different samples. Dotted lines represent identical measurements in the presence of 10 μM CCCP. One division of the *y* axis corresponds to 0.1 pH unit.

What determines the inward proton transport activity of D217E ASR? Aspartate and glutamate have similar properties, but the proton transport activity was much higher for glutamate. The absorption spectra of WT and D217E ASR are similar (Figure 4A), and HPLC analysis showed that the all-trans form of retinal is dominant in both dark-adapted samples (Figure 4B).[13] The similarity of D217E to WT ASR in the unphotolyzed

(15) The observed inward proton transport can be straightforwardly interpreted as a light-driven inward proton pump. Nevertheless, an inward cation "pump" has to be carefully defined in sphaeroplast vesicles, because the interior of the cell is negatively charged. An ion pump is a protein that transports ions against the electrochemical potential (uphill reaction), whereas the observed inward proton transport may be driven along the potential gradient (downhill reaction). Since the electrochemical potential for protons in the present sphaeroplast vesicles is unclear, we have used the term "inward proton transport" rather than "inward proton pump" in this article. On the other hand, the FTIR results for the ASR sample (Figure 5) are coincident with the proton transport activity, suggesting that the inward proton transport is an intrinsic property of D217E ASR. See footnote 18 for further details.

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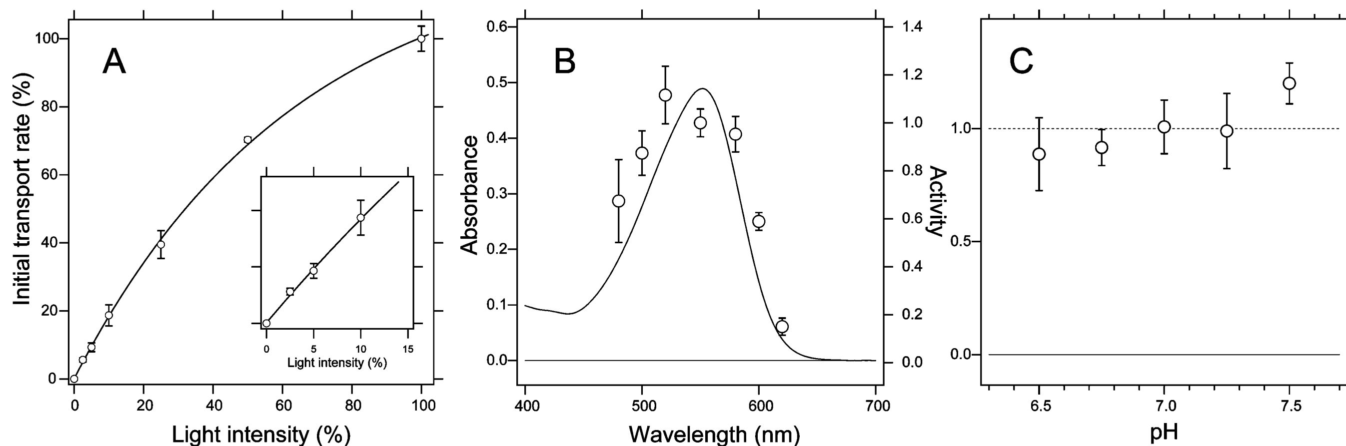

Figure 3. (A) Dependence of the initial rate of proton transport of D217E ASR on light intensity. The inset expands the region of light intensity between zero and 15%. (B) Spectral coincidence of the proton transport activity and absorption of D217E ASR. Open circles represent proton transport activity upon illumination with monochromatic light by use of an interference filter, and the solid line shows the absorption spectrum of the dark-adapted form of D217E ASR. (C) pH dependence of the proton transport activity of D217E ASR.

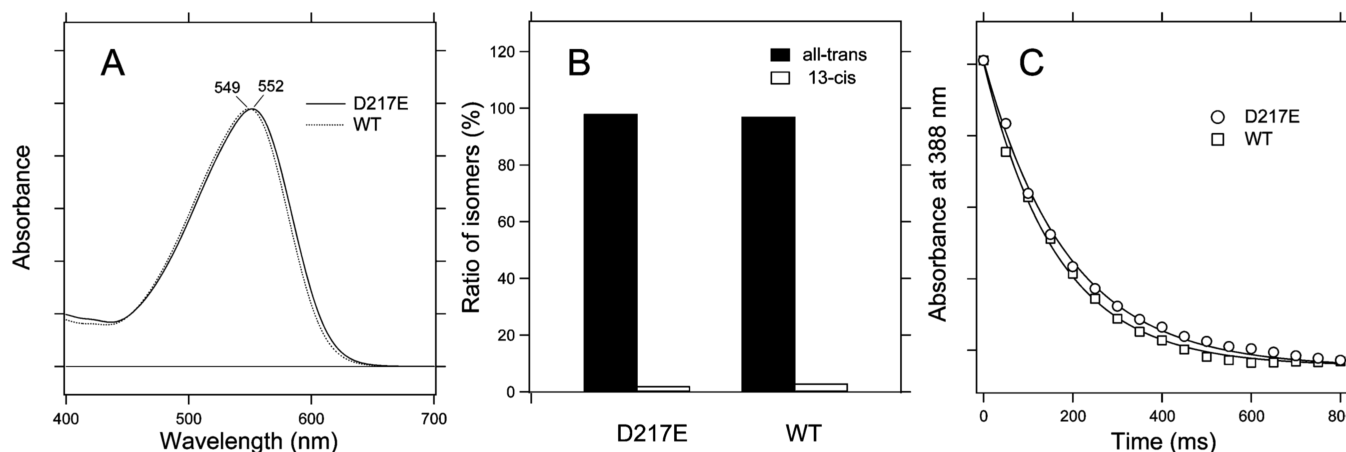

Figure 4. Molecular properties of WT and D217E ASR, which were measured in a DM solution at pH 7.0. (A) Absorption spectra of the dark-adapted form of WT (dotted line) and D217E (solid line) ASR. (B) Isomeric composition of the retinal chromophore in the dark-adapted form. WT and D217E ASR possess 97 and 98% all-trans forms, respectively. (C) Decay kinetics of the M intermediate measured at 388 nm and 277 K. The time constants for WT and D217E ASR were 162 and 188 ms, respectively.

state is reasonable because Asp217 is located ∼15 Å from the retinal chromophore (Figure 1B). The M intermediate state is similarly formed for D217E ASR, whose decay was also similar to the case for the WT (Figure 4C).

The light-induced M-minus-ASR difference FTIR spectra for WT and D217E were also similar (Figure 5), but a remarkable difference is seen at 1760−1700 cm^{-1}, the characteristic frequency region of protonated carboxylic acids (C=O stretching vibration of the COOH group). The spectrum of the WT (Figure 5, top panel) exhibits a broad positive band at 1740−1700 cm^{-1}, and Shi et al.[7] interpreted this feature as evidence for protonation of Asp217 in the M intermediate. A stronger positive peak is observed at 1713 cm^{-1} for D217E ASR, which is down-shifted in D_2O (Figure 5, bottom panel). These observations suggest that the Schiff base proton is transferred to Glu217 in the M intermediate. It should be noted that the spectra of WT and D217E ASR are normalized to the retinal bands at 1250−1200 cm^{-1} (Figure 5), indicating that the same amount of ASR was converted to the M state for both the WT and D217E. Nevertheless, protonation of Glu217 was about 10 times larger than that of Asp217 in the WT. This is completely coincident with the proton transport activity (Figure 2). It is thus likely that the proton affinity at position 217 is correlated with the inward proton transport activity.[18]

On the basis of the present FTIR data, the mechanism of the inward proton transport in D217E ASR can be explained as follows. M formation accompanies deprotonation of the Schiff base, and Glu217 acts as a proton acceptor in D217E ASR. Figure 2 suggests that the pK_a of Glu217 is lower than 6.5 in the unphotolyzed state but higher than 8.0 in M. In BR, the proton acceptor is Asp85 in the extracellular side,[2] and the Schiff base nitrogen interacts with the side-chain oxygen (at a distance of 4.4 Å[1]) through a strongly hydrogen-bonded water in the unphotolyzed state (Figure 1A).[19] Interestingly, ASR also has a negatively charged Asp75 at 3.5 Å,[6] but the Schiff base proton is transferred to Glu217 that is far distant (∼15 Å) (Figure 1B).

(18) In the FTIR measurements, detergent-solubilized ASR molecules were reconstituted into PC liposomes. Therefore, there was no electrochemical gradient for the ASR sample, in contrast to the situation for those in sphaeroplast vesicles. Figure 5 clearly shows that protons were transferred to Glu217 under such conditions, which is completely coincident with the proton transport activity of D217E ASR. This observation suggests that D217E ASR is an inward proton pump. However, the active transport has to be measured to obtain direct evidence of an inward proton pump.

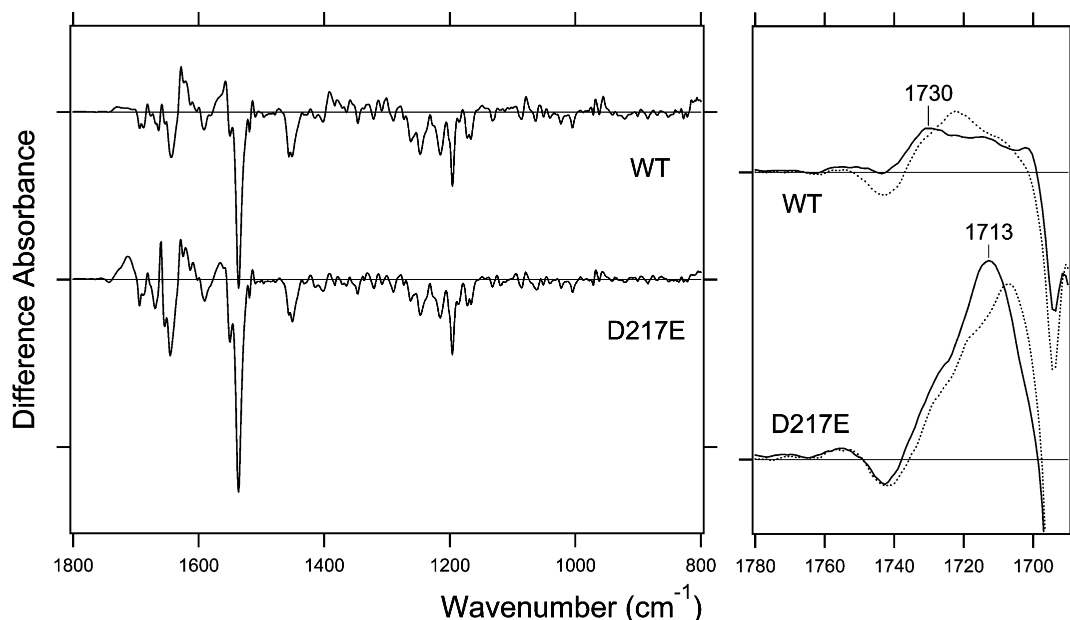

Figure 5. (Left) Light-minus-dark difference IR spectra of WT and D217E ASR in the 1800−800 cm^{-1} region. The spectra were measured for PC liposomes at pH 9.0 and 230 K. One division of the *y* axis corresponds to 0.02 absorbance units. (Right) The spectra are highlighted at the frequency region of protonated carboxylic acids (1780−1690 cm^{-1}). Solid and dotted lines correspond to the measurements in H_2O and D_2O, respectively. One division of the *y* axis corresponds to 0.004 absorbance units.

This suggests that the accessibility of the Schiff base just before release of the proton is toward the cytoplasmic side in ASR but toward the extracellular side in BR. This may suggest the important role of Asp212 in BR, the second negative charge in the Schiff base region, which is replaced by Pro in ASR (Figure 1).

We infer that the Schiff base proton is transferred both inwardly and outwardly upon M formation in ASR, because Asp75 does not act as the proton acceptor.[7,20] This view is also consistent with the previous photocurrent results,[8] though the effect of truncation was not reproduced in the present study. The proton acceptor at the extracellular side is unclear, and it is possible that the Schiff base proton is released into the extracellular aqueous phase. The proton acceptor at the cytoplasmic side is Asp217, but the small FTIR signal of the WT (Figure 5) suggests that the proton is not fully occupied by Asp217. The hydrogen-bonding network in the cytoplasmic domain may accept the Schiff base proton,[21] or it may be released into the cytoplasmic aqueous phase. During the relaxation process, the Schiff base is reprotonated from the same sides of release in WT. In contrast, the strong proton affinity of Glu217, not Asp217, presumably yields reprotonation from the extracellular side in D217E ASR. Thus, ASR does not possess the specific switch mechanism of the BR-like outward proton pump,[22−25] but inward proton transport was clearly observed.

For BR-like proton pumps, outward vectoriality is particularly important for the creation of a proton gradient. It has been inferred that ancestral rhodopsin functioned as a light-driven outward proton pump.[26] In fact, the archaeal-type photosensors, SRI and SRII, pump protons outward in the absence of transmembrane transducer protein.[27,28] During evolution, conversion into inward proton pumps must be strongly prohibited, because it is dangerous for survival. In view of this, why can ASR be easily converted into an inward proton transport? We infer that ASR became a light sensor that activates a soluble transducer protein, and the hydrogen-bonding network in the cytoplasmic domain and its changes must be important for the activation.[21] It is the symmetrical hydrogen-bonded network from the Schiff base (Figure 1B) that allowed creation of an inward proton transport by a single amino acid replacement.

The newly designed inward proton transport may be useful as an application tool in cell biology. The recent discovery of channelrhodopsin, with a light-activated cation channel, has allowed numerous applications in neurobiology, because transport of cations can be triggered by light.[29] Currently, channelrhodopsin and halorhodopsin, a light-driven inward chloride pump, are used as neuroengineering tools to investigate neural circuit function.[30] The newly designed inward proton transport (D217E ASR, presumably acting as a pump) could provide another kind of active control of electrochemical potential in cells by light, in contrast to channelrhodopsin of a purely passive nature. Another application of this protein may be in the field

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of acidosis-induced cell death. Intracellular pH is precisely regulated at ∼7.2 by various transporters, which may be changed by acidification around tumor cells (6.9−7.0). Thus, tumor metabolism and pH-control systems have been targets for novel anticancer therapies.[31] Acidification of cells by light using an inward proton transport will be useful in the research field. For these applications, a more efficient proton transport may be required, because the current efficiency of D217E ASR is 15 times lower than that of BR, and an additional mutation study is in progress.

In conclusion, the present study created a light-driven inward proton transport from ASR, a bacterial photochromic sensor protein, by a single amino acid replacement. Native ASR has only small proton transport activity, but a mutation of Asp217 to Glu significantly increases the inward proton transport activity. FTIR spectroscopy clearly detected the protonation signal of Glu217 but not of Asp217 in WT ASR. The strong proton affinity of the acceptor on the cytoplasmic side appears to force proton uptake from the extracellular side after the Schiff base deprotonation, even though the carboxylate is 15 Å distant from the retinal Schiff base.

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